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Contraction, Serotonin-Elicited Modulation, and Membrane Currents of Dissociated Fibers of *Aplysia* Buccal Muscle

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Abstract. Feeding muscles of the buccal mass of *Aplysia* are innervated by cholinergic and serotonergic neurons. Buccal muscle I5 contracts in response to acetylcholine (ACh). During feeding arousal, ACh-elicited contraction of muscle I5 is potentiated by serotonin (5-HT). This paper demonstrates a dissociated cell preparation of muscle I5 in which cellular mechanisms regulating contraction can be investigated.

Dissociated muscle fibers contracted in response to both KCl and ACh. Serotonin (10^{-6} *M*) significantly potentiated the shortening caused by both KCl and ACh. Potentiation lasted at least 4 min, similar to potentiation in intact muscles.

Four types of currents recorded by patch clamp methods are illustrated. With 540 m*M* KCl in the patch electrode, stretch-activated channels having a chord conductance of 150 pS are observed in on-cell patches. In whole cell configuration, ACh elicits inward current at a holding potential of −60 mV. With high potassium in the electrode, depolarization elicits an outward current. The voltage-dependent outward current is blocked with cesium in the electrode and 4-aminopyridine and tetraethylammonium outside the cells. The remaining voltage-dependent inward current is calcium dependent. The voltage-dependent inward and outward currents are activated within the range of depolarization produced by ACh and may therefore play roles in regulating contractile responses elicited by ACh.

Introduction

Neurotransmitters can cause both direct and modulatory effects on target tissues. A "modulatory effect" of a neurotransmitter is one in which the transmitter has no immediate effect, but rather, it modifies the influence of other effectors. These modulatory influences appear to fine-tune the nervous system and the muscles it controls for switching between different behavioral tasks. The same circuits can subserve several different behaviors; the behavioral output depends on the relative weighting of different synapses and the excitability of circuit elements. Modulation of muscle is part of this integrated scheme. Although the relative strength of contractions appropriate for different behaviors could be manipulated by discrete relative changes in motoneuron activity, an additional, possibly more efficient method may be to give different "global commands" that change the relative strengths of contractile responses in ways appropriate for particular behaviors. An example of a modulatory effect of a neurotransmitter mediating a change in behavior is the potentiating effect of serotonin (5-HT) on buccal mass muscles of *Aplysia*, which is believed to mediate, in part, feeding arousal.

The buccal mass muscles of *Aplysia* are smooth muscles used in voluntary feeding movements. These muscles are innervated by cholinergic, peptidergic, and serotonergic neurons. Acetylcholine (ACh) is the direct effector of these muscles, as exemplified by the contractile response to ACh of buccal muscle I5. Muscle I5, also known as the accessory radular closer muscle (Cohen *et al.*, 1978), is the most intensively studied *Aplysia* muscle. I5 is innervated by buccal ganglion neurons B15 and B16 (Cohen *et al.*, 1978; Ram, 1983), both of which synthesize ACh (Cohen *et al.*, 1978) as well as several peptides (Cropper *et al.*, 1987, 1988). In addition, I5 is innervated by the serotonergic metacerebral giant cell (MCG). Activity of MCG causes no direct response of the muscle; however, it potentiates subsequent contractile responses to B15 and B16. This modulation is achieved largely through post-synaptic actions on the muscle (Weiss *et al.*, 1978). In isolated I5

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Figure 1. Typical dissociated buccal muscle fiber. At the right is a patch electrode pointing to the fiber. Scale: calibration marks are 7.8 μm apart.

muscles 5-HT produces no contractile response itself but does potentiate ACh-elicited contractions (Ram *et al.*, 1981). MCGs are active during feeding (Weiss *et al.*, 1978). Lesion of serotonergic neurons changes (although does not completely block) feeding motor activity (Rosen *et al.*, 1983, 1989). Thus, the modulatory effect of the serotonergic MCG neurons on feeding muscles appears to have an important role in feeding arousal.

Although previous experiments on mechanisms mediating the modulatory effect of 5-HT on buccal muscles have suggested roles for cyclic AMP (Mandelbaum, 1980; Ram *et al.*, 1983, 1984a) and calcium (Ram *et al.*, 1984b; Ram and Parti, 1985), the cellular targets of these mediators have not been determined. For example, it is unknown whether cyclic AMP or calcium modify membrane mechanisms such as ion channels or change the sensitivity or activity of contractile proteins. For studying effects on contractile proteins, this laboratory developed a skinned muscle preparation, which is described elsewhere (Ram and Patel, 1989). To study membrane mechanisms, we developed a dissociated muscle fiber preparation. This paper describes the contractile properties of this dissociated muscle preparation, including its modulatory response to 5-HT, and demonstrates its suitability for patch clamp analysis of single channel and whole cell ionic currents. Preliminary descriptions of some of these data have appeared previously (Ram and Liu, 1990; Zhang and Ram, 1990).

Materials and Methods

Individuals of *Aplysia californica* (200–400 g) were obtained from Marinus (Long Beach, California) and maintained at 18°C in Instant Ocean with a 12:12 L:D light cycle. To obtain dissociated muscle fibers, a modification of the methods of Ishii *et al.* (1986), previously used to dissociate muscle fibers in *Mytilus*, was used. Both I5 buccal muscles were dissected from the animal, teased into thin strips, and incubated for 2–4 h at 28°C in Instant Ocean containing 10 m*M* HEPES (pH 7.0, adjusted with NaOH), 0.15% collagenase (Sigma Type I), 0.1% soybean trypsin inhibitor (Sigma Type I-S), and 1 μg/ml leupeptin

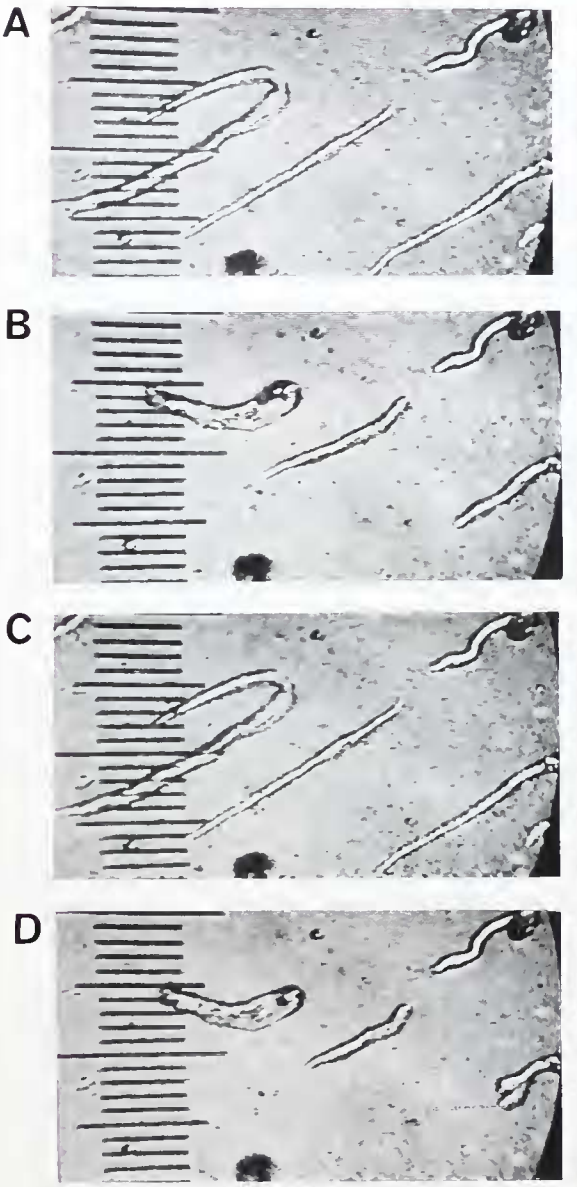

Figure 2. Serotonin (5-HT) potentiates high potassium-elicited contraction. Dissociated muscle fibers in a small chamber (0.4 ml volume) were constantly superfused with artificial seawater (ASW) at 5.5 ml/min. (A) Fibers at rest. (B) Contractile response to a 3-s pulse of ASW containing 100 m*M* KCl. All but the fiber in the upper right contracted. Fibers returned to rest length at the end of the pulse of KCl. (C) Fibers at the end of 1 min superfusion with ASW containing 10^{-6} *M* 5-HT. Fibers remained at rest length. (D) Contractile response to a 3-s pulse of 100 m*M* KCl ASW, identical to that given in (B), immediately after 1 min superfusion with 10^{-6} *M* 5-HT ASW. All contracting fibers shortened more after 5-HT treatment. Scale: calibration marks are 12 μm apart.

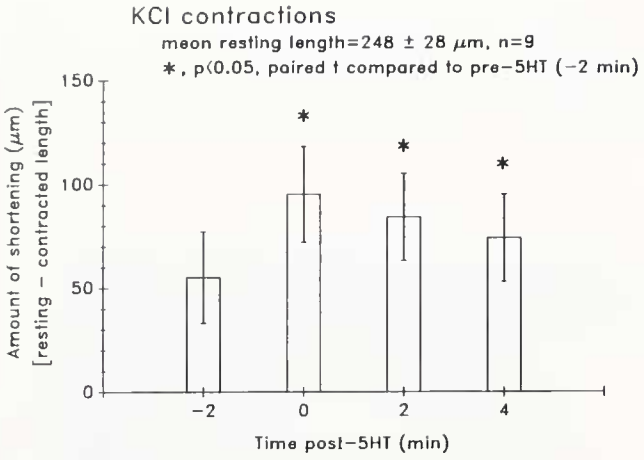

Figure 3. Magnitude and time course of 5-HT potentiation of KCl-elicited contractions. Fibers were measured in images similar to those illustrated in Figure 2. High potassium (100 m*M* KCl ASW) pulses were given every two min. Fibers were exposed to 5-HT (10^{-6} *M* in ASW) for 1 min immediately prior to the 0 time point.

(Sigma). When cells began appearing in the medium, fibers in remaining muscle pieces were dispersed by gentle trituration. The resultant dissociated cells were washed by centrifuging and resuspending them in wash medium containing all ingredients of the dissociating medium except collagenase. Washed cells were plated onto glass coverslips in 30-mm plastic petri dishes and stored at 4°C in a humidified chamber. Cells were usually used within 1–4 days, although viable, contractile cells have survived for as long as 10 days under these conditions. Experiments were done at room temperature (20–24°C) after allowing the cells to warm gradually for at least 30 min.

A plexiglass insert having a central hole approximately 1 cm in diameter was clamped into the 30-mm petri dish. The insert formed a small chamber, approximately 0.4 ml in volume. Medium was constantly pumped into the chamber (5.5 ml/min) and removed by suction from a surface wick opposite the inflow. The dish was mounted on a movable stage of an inverted microscope. The shape and movement of muscle fibers were recorded by a VHS camcorder (RCA CC310). A videotape demonstrating many of the contractile and electrophysiological responses reported in this paper ("Dissociated Muscle Fibers of *Aplysia*," by J. L. Ram) is available from the authors upon request. Morphometric analysis was done by measuring still-images on the tape playback. Photographs were made by oscilloscope camera directly off the TV monitor.

A Dagan 8900 Patch Clamp-Whole Cell Clamp was used for single channel and whole cell recording. Data were filtered by the 1 kHz low-pass filter in the Dagan amplifier. Electrodes were fabricated from Fisher non-heparinized hematocrit glass, polished to bubble number 3–4 (Corey and Stevens, 1985), and coated with Sylgard. Pipet solutions are described in relevant figure captions. Electrical stimuli and digital recording of currents were controlled by pCLAMP software (Axon Instruments, Burlingame, California).

Results

Morphology and contraction

The typical appearance of a dissociated fiber from buccal muscle I5 is illustrated in Figure 1. Dissociated muscle fibers were spindle-shaped and ranged from 5 to 25 μm in diameter and up to a mm in length. The widest diameter usually occurred near the middle of the fiber, adjacent to the nucleus, and averaged 13.6 ± 0.9 μm (mean ± S.E., n = 19). The average diameter of the fibers, measured every 20 μm along the length of the fiber, was 10.8 ± 0.7 μm. The average length of fibers at rest was 270 ± 10 μm (n = 37).

Dissociated muscle fibers contracted in response to KCl. In response to a 2- or 3-s pulse of ASW containing 100 m*M* KCl, 22 fibers that had average resting lengths of 262 ± 13 μm shortened to 218 ± 12 μm. An illustration of a subset of these fibers is shown in Figure 2, in which Figure 2A shows the fibers at rest and Figure 2B shows the max-

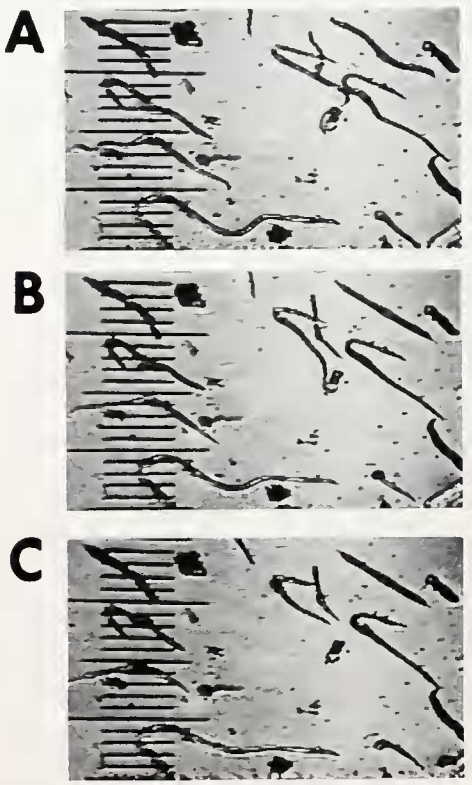

Figure 4. Serotonin (5-HT) potentiates ACh-elicited contractions. The procedure is identical to that of Figure 2 except that contraction was elicited by a 3-s pulse of 10^{-4} *M* ACh. (A) Fibers at rest. (B) Contractile response to ACh. (C) Contractile response to identical pulse of ACh as in (B) immediately after 1 min superfusion with 10^{-6} *M* 5-HT ASW. Scale: calibration marks are 20 μm apart.

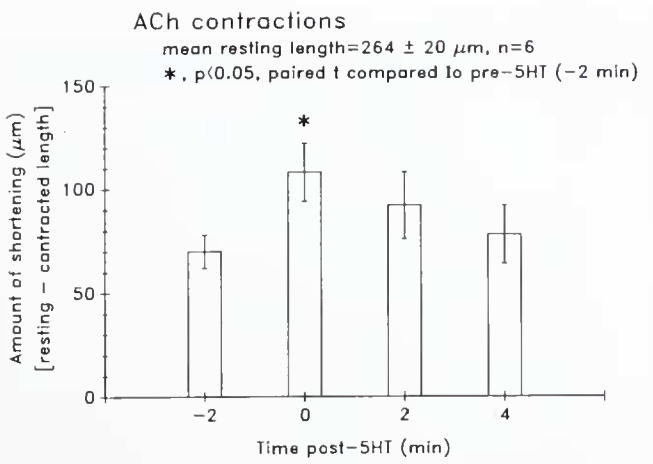

Figure 5. Magnitude and time course of 5-HT potentiation of ACh-elicited contractions. Fibers were measured on the field in Figure 4 and subsequent images. ACh pulses were given every 2 min. Fibers were exposed to 5-HT (10^{-6} *M* in ASW) for 1 min immediately prior to the 0 time point.

imal contraction produced by a 3-s pulse of KCl. Following the KCl pulse, fibers relaxed to their resting lengths within a few seconds.

Serotonin potentiated the contractile response to KCl (Fig. 2D). Figure 3 summarizes data from nine fibers that were exposed to 10^{-6} *M* 5-HT for 1 min. The amount of shortening produced by KCl pulses was almost doubled following 5-HT, and the effect lasted at least 4 min, similar to the long-lasting potentiation in intact muscles produced by 5-HT (Ram *et al.*, 1981). The fibers remained relaxed during the 5-HT application (Fig. 2C).

Similarly, ACh caused contraction of dissociated fibers, which could be potentiated by 5-HT. Figure 4 shows ACh-elicited contractions prior to 5-HT and immediately following one min 10^{-6} *M* 5-HT. Data from six fibers, summarized in Figure 5, show the significant increase in shortening caused by 5-HT and the similar time course of recovery from the effects of 5-HT to KCl-elicited contractions.

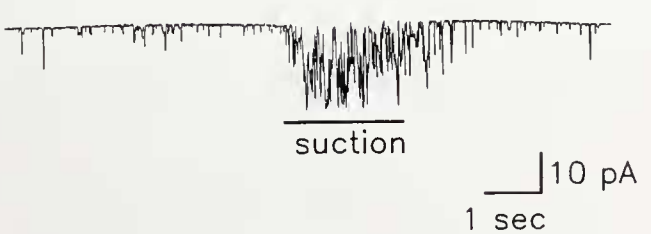

Figure 6. Channel activity in on-cell patches. The electrode contained (in m*M*) 406 KCl, 20 NaCl, 2 $MgCl_2$, 10 ATP, 0.1 GTP, 10 glutathione, and 100 HEPES, pH 7.0 with KOH. Electrode potential was identical to the bath potential, and the cell was at resting potential (not measured for this cell). Suction increased the opening of at least one population of large channels, conducting approximately 10 pA per unitary channel opening.

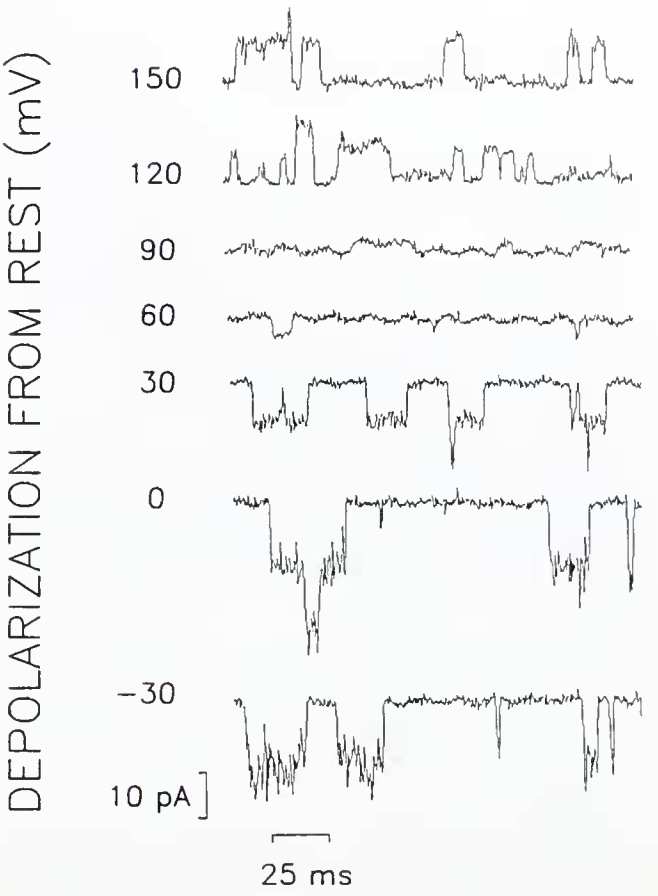

Figure 7. Current through stretch-activated channels in an on-cell patch, measured over a range of membrane potentials. The electrode contained 540 m*M* KCl. Stretch sensitivity of these channels was demonstrated during another part of the experiment (not illustrated here). Membrane potential was varied by changing the potential of the patch electrode, and the membrane potential is given as the change from resting potential. Pipet potential was held at each potential for at least 20 s. Channel current reversed at approximately 87 mV; chord conductance was approximately 150 pS; and channel opening probability was independent of membrane potential.

Single channel and whole cell patch clamp recording

Dissociated buccal muscle fibers were suitable for forming gigaseals for single channel and whole cell recording. With high potassium in the patch electrode, on-cell patches revealed the presence of a variety of channels conducting inward current at resting potential, including at least one prominent channel that could be activated by increased suction on the electrode (Fig. 6). The frequency of the opening of suction-activated channels increased with negative pressures of 50–100 cm H_2O (approximately 40–80 mm Hg), as measured by a water manometer (n = 3; see also Ram *et al.*, 1990). The current through unitary channel openings of the most prominent channel activated by suction, with the fiber at resting potential and the electrode at bath potential, averaged 12 ± 1 pA (n = 7 patches). The chord conductance of this

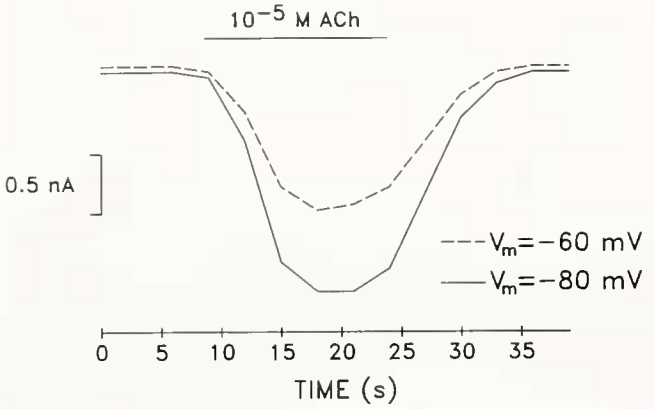

Figure 8. Response to ACh in whole-cell recording configuration. Pipet solution contained (in m*M*) 20 NaCl, 406 KCl, 2 $MgCl_2$, 100 HEPES, 5 EGTA, 5 MgATP, 0.1 NaGTP, 10 glutathione, pH 7.0 (adjusted with KOH). Fibers were constantly superfused with artificial seawater. Holding potential was −80 mV and was moved to −65 mV every 3 s. A 15-s pulse of 10^{-5} *M* ACh elicited inward current at both membrane potentials.

suction-activated channel, determined by eliciting channel activity at several different holding potentials, averaged 140 ± 20 pS (n = 3), as exemplified by the patch illustrated in Figure 7.

After gigaseal formation, whole cell configuration could be achieved by applying greater suction than is necessary to activate suction-activated channels. With a pipet solution containing high potassium and other ingredients meant to mimic the normal intracellular milieu of the fibers (complete composition is given in the caption to Fig. 8), ACh elicited an inward current (Fig. 8). The peak current elicited by 10^{-5} *M* ACh at a holding potential of −80 mV was −2.4 ± 0.5 nA (n = 20); at a holding potential of −60 mV, the peak current averaged −1.4 ± 0.4 nA (n = 20). Under the same ionic conditions, depolarization activated outward current (Fig. 9). In observations of more than 20 cells, a net voltage dependent inward current was never seen under conditions of having high potassium in the pipet and normal sea water outside. Occasionally, there was a slight delay in activation of outward current (not seen in Fig. 9), possibly indicating an initial counterbalancing inward current.

Voltage-dependent inward current can, however, be seen under conditions that block potassium channels. With cesium in the electrode and 4-aminopyridine and tetraethylammonium in the extracellular solution, depolarization elicited an inward current. The peak inward current averaged 2.4 ± .4 nA (n = 11 fibers). As illustrated in Figure 10, the voltage dependent inward current was dependent upon calcium in the extracellular medium.

Discussion

This paper demonstrates that smooth muscle fibers dissociated from buccal muscles of *Aplysia* are a suitable preparation for studying mechanisms regulating contraction and its modulation. First, isolated fibers have appropriate contractile responses: They contract in response to both high potassium and ACh, and the contractions to both are potentiated by 5-HT. Second, the dissociated fibers are suitable for patch clamp analysis of single channel and whole cell currents.

Previous studies have used indirect methods for investigating the roles of specific ion channels in regulating contraction of molluscan muscles. One set of questions that arise concerns the sources of activator calcium in the physiological responses to neurotransmitters. Is contraction dependent upon the influx of extracellular calcium? If so, are the channels receptor operated or voltage dependent, and are they specific for calcium? Many molluscan muscles are highly dependent upon extracellular calcium to trigger contraction. For example, ACh-elicited contractions of buccal muscle E1 (another muscle of the *Aplysia* buccal musculature whose contraction is potentiated by 5-HT) fail within two minutes of removal of extracellular calcium (Ram *et al.*, 1984b). Similarly, calcium-dependence of ACh-elicited contractions have also been demonstrated in a non-spiking muscle of *Aplysia* gill (Reilly and Peretz, 1987) and in four different pro-

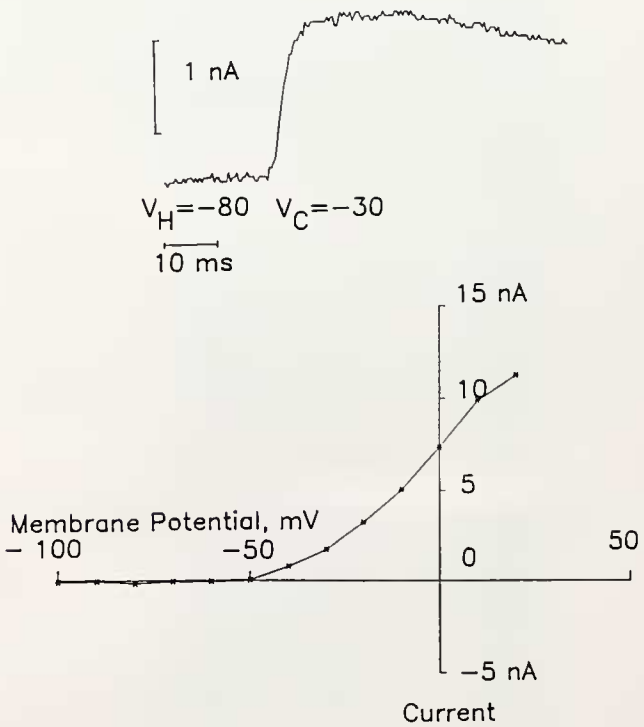

Figure 9. Voltage-dependent outward current in whole-cell configuration. Pipet solution and external medium were the same as in Figure 8. Holding potential was −80 mV. Currents were elicited by 60-ms pulses to various potentials, from −100 mV to +20 mV, given at intervals of 1.2 s. Linear leak and capacitative transients have been subtracted. (Upper) Typical response to −30 mV. (Lower) Current voltage relationship for peak current during the pulse.

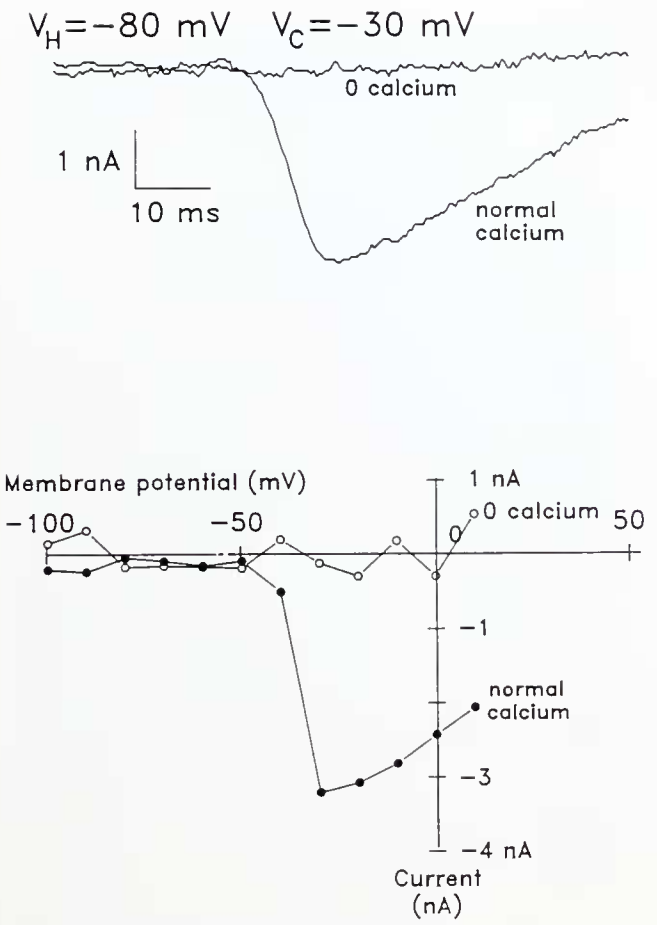

Figure 10. Voltage-dependent calcium current in whole-cell configuration. Pipet solution contained (in m*M*) 20 NaCl, 406 CsCl, 2 $MgCl_2$, 100 HEPES, 5 EGTA, 5 MgATP, 0.1 NaGTP, 10 glutathione, pH 7.0 (adjusted with CsOH). External medium was artificial seawater containing (in m*M*) 50 tetraethylammonium, 5 4-aminopyridine, 485 NaCl, 25 $MgSO_4$, 25 $MgCl_2$, 10 KCl, 10 $CaCl_2$, and 10 HEPES, pH 7.8 (adjusted with NaOH). For 0 calcium medium, the $CaCl_2$ was left out and all other ingredients increased in concentration by 1%. Holding potential was −80 mV. Currents were elicited by 60 ms pulses to various potentials, from −100 mV to +10 mV at intervals of 1.2 s. Linear leak and capacitative transients have been subtracted. (Upper) Typical responses to depolarization to −30 mV in the presence and absence of calcium. (Lower) Current-voltage relationships for peak current in the presence and absence of calcium.

boscis muscles of the marine snail *Busycon* (Huddart and Hill, 1988; Huddart *et al.*, 1990a, b; Hill and McDonald-Ordzie, 1979; Hill *et al.*, 1970). Another molluscan muscle, the anterior byssus retractor muscle (ABRM) seems less dependent upon extracellular calcium because ACh-elicited contractions of ABRM are not abolished by the removal of extracellular calcium for up to 10 min (Sugi and Yamaguchi, 1976). Recent investigations of intracellular calcium levels in ABRM using the calcium-sensitive fluorescent indicator FURA-2 have shown that although extracellular calcium may account for the majority of the rise in intracellular calcium in response to cholinergic activation, about 30% of the increase in intracellular free calcium may be attributed to the release of stored calcium (Ishii *et al.*, 1988).

Studies on the influx of calcium-45 in response to ACh have been used to determine whether calcium dependence of contraction involves physical movement of calcium into the cell. ACh stimulates influx of calcium-45 into *Aplysia* buccal muscles E1 and I5 (Ram and Parti, 1985; Gole *et al.*, 1987); however, ACh does not significantly increase calcium-45 influx into ABRM (Tameyasu and Sugi, 1976). The lack of significant ACh-stimulated calcium-45 influx in ABRM not only contrasts with the observations in *Aplysia* muscles but also stands in apparent contradiction with FURA-2 measurements showing a significant extracellular dependence of the ACh-stimulated rise in intracellular calcium in ABRM (see above). Calcium-45 influx measurements are inherently more variable than FURA-2 measurements. Therefore, the lack of significant effect of ACh on calcium-45 influx in ABRM probably reflects a relatively lower importance of calcium influx in ABRM compared to *Aplysia* buccal muscles rather than a complete absence of ACh-stimulated influx.

A possible route for calcium entry into muscle cells during the response to ACh is via voltage-dependent calcium channels. As discussed below, ACh causes depolarization of molluscan muscles. Previous evidence that depolarization could activate voltage dependent calcium channels included demonstrating that another depolarizing stimulus, a high potassium medium, causes contraction. High potassium induces contractions of *Busycon* proboscis retractor muscles (*e.g.*, Huddart *et al.*, 1990a, b), ABRM (*e.g.*, Twarog and Muneoka, 1972), and *Aplysia* buccal muscles (Ram, unpub. data). Furthermore, high potassium elicits contraction of isolated fibers, as described in *Aplysia* buccal muscle (Figs. 2 and 3, this paper) and in ABRM (Ishii *et al.*, 1986), unambiguously proving that contraction elicited by high potassium is a direct effect on single fibers and is not dependent upon either release of neurotransmitters from nerve endings in the muscle or mechanical or electrical coupling between fibers. Ishii *et al.* (1988) has also used FURA-2 to show that high potassium causes an increase in intracellular calcium that is completely dependent upon extracellular calcium.

Blockers of voltage-dependent calcium channels reduce the contractile responses produced by both ACh and high potassium. Thus, Huddart *et al.* (1990b) found that diltiazam, verapamil, and nifedipine all decrease ACh-elicited contractions of *Busycon* proboscis muscles. Similarly, nifedipine reduces ACh-elicited contraction of *Aplysia* buccal muscle (Ram and Liu, 1990).

The above indirect evidence for the existence of voltage-dependent calcium channels is now supported in the present paper by voltage clamp recordings of a voltage-dependent inward current that is dependent on extracellular calcium (Fig. 10). As described in a preliminary re-

port (Ram and Liu, 1990), we have also demonstrated that this current is partially inhibited by nifedipine and completely blocked by lanthanum.

For voltage-dependent calcium channels to play a role in mediating ACh responses, it must also be shown that the range of membrane potentials at which a voltage-dependent calcium current can be activated is within the range of membrane potentials caused by ACh. As illustrated in Figure 10, voltage-dependent calcium current begins to activate with depolarizations to −40 mV. In other cells (data not shown), voltage-dependent calcium current has been activated with depolarization to as little as −50 mV. As discussed below, ACh can depolarize cells to approximately −35 mV. Thus, the membrane potential required to activate voltage-dependent calcium channels is clearly within the range of depolarization produced by ACh in *Aplysia* buccal muscles. This paper provides the strongest evidence yet that activator calcium enters molluscan muscle fibers during cholinergic stimulation by voltage-dependent calcium channels.

This paper also initiates the analysis of receptor-operated channels activated by ACh. ACh causes depolarization of molluscan muscle fibers in clam heart (Wilkens and Greenberg, 1973), ABRM (Twarog, 1954), *Busycon* proboscis muscles (Huddart *et al.*, 1990b; Hill and Licis, 1985; Hill and McDonald-Ordzie, 1979), *Aplysia* gill muscle (Reilly and Peretz, 1987) and *Aplysia* buccal muscles (Ram *et al.*, 1990). Detailed quantitative studies of *Aplysia* buccal muscle revealed (a) an average resting potential of −65 mV (Gole *et al.*, 1987), (b) no contraction elicited with depolarizations less than approximately 10 mV above rest, (c) a non-linear relationship between contraction and depolarization in which increasing ACh beyond a certain concentration was accompanied by increasing contraction with little or no further increase in depolarization, and (d) maximal ACh-elicited depolarization of approximately 30 mV above rest (Ram *et al.*, 1990).

The limit on depolarization produced by ACh to only 30 mV above rest may result from several mechanisms. One possibility is that the reversal potential for ACh-activated channels is only 30 mV above rest. An alternative explanation is that depolarization activates voltage-dependent potassium channels that act as an effective brake on further depolarization even in the face of activation of more ACh-activated channels. Data in this paper show that analysis of this question is feasible. As expected for a depolarizing stimulus, ACh activates inward current (Fig. 8). The reversal potential of the ACh response is under investigation (Ram and Liu, 1990). Furthermore, Figure 9 demonstrates that the voltage-dependent outward current is activated within the voltage range elicited by ACh. The voltage-dependent outward current is undoubtedly potassium because it is blocked by TEA and 4-AP outside the cell and Cs in the electrode (Fig. 10).

This paper also demonstrates that buccal muscle fibers contain stretch-activated channels. Because these channels were observed with on-cell patch electrodes containing only KCl, the inward currents illustrated are almost certainly due to potassium current. Similarly, stretch-activated channels conducting primarily potassium ions have been reported previously in molluscan neurons (Morris and Sigurdson, 1989; Sigurdson and Morris, 1989) and cardiac muscle (Brezden and Gardner, 1986; Sigurdson *et al.*, 1987). Stretch-activated channels that are somewhat less selective for potassium have been reported in various mammalian tissues, including skeletal muscle (Guharay and Sachs, 1984) and smooth muscle (Kirber *et al.*, 1988).

In future experiments it should be possible to determine whether 5-HT potentiates contraction of dissociated muscle fibers by modifying the ionic currents illustrated here. One indication in a molluscan muscle that 5-HT may change membrane currents of molluscan muscles is that, in ABRM, 5-HT potentiates the rise in intracellular calcium caused by 100 m*M* KCl (Ishii *et al.*, 1989). The rise in intracellular calcium in response to KCl is completely dependent upon extracellular calcium (Ishii *et al.*, 1988) and is presumed to be due to voltage-dependent calcium current, similar to the current described in this paper in Figure 10. In addition, 5-HT might also be modifying potassium channels, receptor-operated channels, and stretch-activated channels.

Acknowledgments

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